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Abstract

By subcutaneous inoculation of N, N'-dimethylnitrosourea to adult male C3Hf/Bi mice once a week for 10 consecutive weeks the authors studied the correlation between immunological functions and histological changes in lymphatic tissues at the latent period of thymic lymphoma whose development is known to occur in 100 per cent. As a result, it was found that PFC of the spleen to sheep erythrocytes decreased to about one third the normal level by two weeks, and to one tenth by 8 weeks after initial inoculation of this compound. Hemolysin and hemagglutinin titers of the serum became less than 1 : 2 after 6 weeks and later. As for histological changes in the thymus, disappearance of lymphocytes became marked by 2 weeks, and there appeared tumor cells by 8 weeks. Also the peripheral lymphocytes as well as the total spleen cells decreased in number along with increase of the frequency of inoculation of N,N'-dimethylnitrosourea. These results seem to suggest that the immunosuppressive effect of carcinogen facilitates the development and proliferation of tumor cells possessing tumor specific antigenicity in the course of N, N'-dimethylnitrosourea- carcinogenesis.

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RELATIONSHIP BETWEEN IMMUNE REACTIVITY AND DEVELOPEMENT OF THYMIC LYMPHOMA BY N,N′-DIMETHYLNITROSOUREA IN C3Hf/Bi MICE

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Considerable evidences have been accumulated in recent years in support of the hypothesis that depression of the immune response may play a role in the development of tumor (1). Many carcinogenic agents have immunosuppressive activity, for example, x-ray irradiation (2, 3), chemical carcinogens (3, 4, 5), and some of the oncogenic viruses (6, 7, 8, 9). On the other hand, antigenicity specific to tumor cells has been demonstrated in several experimental systems (10). These evidences suggest the possibility that carcinogenic compounds have both activity of cell transformation and of interference with the immunological system, and that such interference facilitates the growth of antigenic tumor cells.

The carcinogenic effects of N, N′-dimethylnitrosourea was first demonstrated by DRUCKREY *et al.* in 1967 (11). They reported a high incidence of neurogenic tumors in BD rats. In the C3Hf/Bi mice, HIRAKI reported that this compound developed thymic lymphoma in 100 % of adult mice by successive subcutaneous injections.

The present investigation was undertaken in order to study the relationship between immunologic capacity and the development of the malignant lymphoma in the C3Hf/Bi mouse inoculated N, N′-dimethylnitrosourea.

MATERIALS AND METHODS

Animal: C3Hf/Bi mice of known age, ranging from 5 to 6 weeks were used. To avoid temporary hormonal variations, only male mice were used.

Administration of N, N′-dimethylnitrosourea: N, N′-dimethylnitrosourea was dissolved in physiological saline solution (PSS). The solution was prepared just before administration and injected subcutaneously into the back. A dose of 80 mg/kg body weight of N, N′-dimethylnitrosourea was given weekly.

Immunization and Serology: Sheep red blood cells (S-RBC) obtained commercially (Kyokuto, Co. Ltd.) were washed three times in PSS by serial centrifuga-

tion (3,000 rpm for 5 minutes) and resuspended in PSS to a concentration of 10% (2.5×10^9RBC/ml).

Test and control mice were immunized by an intraperitoneal inoculation of 0.2ml of the 10% S-RBC. Blood was obtained from individual mice by puncture of the retro-orbital venous plexus. All serum specimens were kept at −20°C until tested. To determine the antibody titer to S-RBC, heat-inactivated (56°C for 30 minutes) serum samples were diluted two-fold serially in the range of 1 : 2 through 1 : 1024. Dilutions were in PSS in 0.05 ml volume.

For hemagglutinin determinations, an equal volume of 0.5 % suspension of freshly washed S-RBC was added to each dilution cup, the microtiter plates were agitated, and incubated for 18 hours at 4°C.

For hemolysin determinations, dilutions were made as described above and a 0.05 ml volume of a mixture composed of equal quantities of 1 % S-RBC and 1 : 40 dilution of commercial dry complement (Kyokuto Co. Ltd.) was added to each serial dilution cup followed by incubation for one hour at 37°C.

Titers were recorded as the reciprocal of the highest dilution of serum which resulted in either complete hemagglutination or hemolysis of the added red blood cells.

Antibody Plaque Assays: The number of hemolytic plaque forming cells (PFC) was determined in spleen of control and test mice with the agar plaque technique, essentially as described by JERNE (13) and CEGLOWSKI *et al.* (9). Four mice per group were killed on 5 days after immunization with S-RBC, and spleen cell suspensions were prepared. A 0.1 ml portion of the cells was added to 1 ml warm melted 0.7 % Noble agar (Difco Laboratories) to which had been added 0.1 ml of 10 % suspension of target erythrocytes and 1.0 mg diethylaminoethyldextran. This mixture was rapidly and carefully poured onto the surface of a 5.5 cm-diameter petri dish containing solidified 1.5 % Noble agar and diethylaminoethyldextran. The dishes were incubated for one hour at 37°C and then treated with 2 ml of a 1 : 40 dilution of commercial dry complement. The dishes were further incubated for a half hour at 37°C until localized and discrete zones of hemolysis appearing on the dishes were stained with H_2O_2-benzidine stain and the plaques were counted. In all instances each spleen cell suspension was tested in duplicate with at least several cell concentrations.

Experimental Procedure: Forty-five male C3Hf/Bi mice, 5–6 weeks old, were inoculated with N, N′-dimethylnitrosourea once a week for 10 successive weeks. Normal mice of similar size were used as controls. At each week, test and control mice 4 each were immunized with S-RBC on the day of inoculation of N, N′-dimethylnitrosourea. On 5 days after immunization white blood cell counts and smear of peripheral blood were made, blood was obtained from each mouse and then mice were sacrificed. The spleen was excised, weighed, teased into Eagle's minimal essential medium, filtered through double gauze and washed three times by serial centrifugation. The number of viable leukocytes was determined and the number of antibody plaque forming cells (PFC) per spleen and per million leukocytes calculated. Histological examinations were made about the thymus, a part of the spleen and lymph nodes.

RESULTS

Cellular and Humoral Responses of Control Mice to S-RBC

Cellular and humoral responses of normal mice to S-RBC were established. Male mice of 6 weeks old were inoculated with the S-RBC and groups of 3 animals each were killed at 1, 3, 4, 5, 6, 7, 10 and 14 days after immunization. A response to S-RBC is shown in Fig. 1. The peak number of PFC appeared on the 4th day after immunization. The number of PFC then declined rapidly and after the curve of the number of PFC continued at a lower plateau. There were nearly 8,000 PFC/spleen and 700/10^6 leukocytes on the 4th day after immunization. (Fig. 1-A, B).

The hemagglutinin and hemolysin titers also increased rapidly after immunization, with titers ranging from 1 : 64～1 : 128 within 4～6 days (Fig. 1-C).

Following these results, when immunological responses were exa-

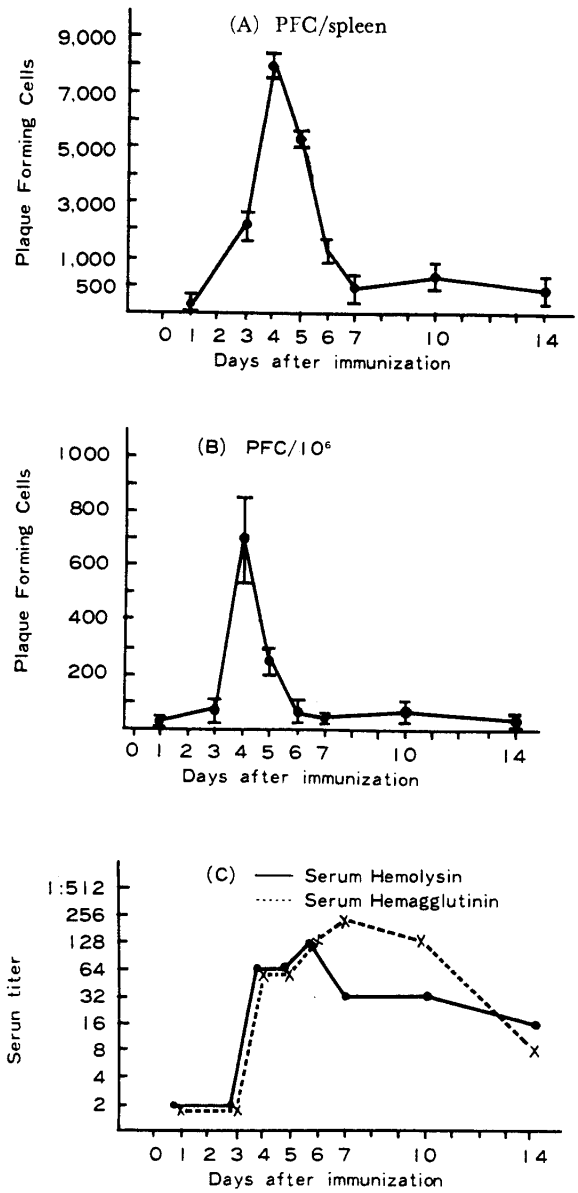

Figure 1. The cytokinetics of the appearance of PFC/spleen (A), PFC/10^6 leukocytes (B), serum hemolysin titers (C, ——) and serum hemagglutinin titers (C, ·····) in normal adult mice immunized with sheep erythrocytes. Each point represents average response of 3 mice. Vertical bars indicate range.

mined 5 days after immunization, humoral and cellular responses could be estimated effectively at the same time. Therefore, the 5th day was chosen for estimating the immunological response in the subsequent experiments.

Cellular and Humoral Responses of Mice Successively Inoculated with N, N'-dimethylnitrosourea

After inoculating 80 mg/kg of N, N'-dimethylnitrosourea successively to mice 5~6 weeks old once a week for 10 consecutive weeks, cellular and humoral responses to S-RBC were observed. As a result, the number of PFC was considerably decreased after two injections of N, N'-dimethylnitrosourea, and by 8 weeks only 500 PFC/spleen and 70 PFC/10^6 leukocytes were caluculated (Fig. 2-A, B). The percentage against the reaction of control animals (percent PFC) seems to be gradually decreased and it was by 2 weeks about 30%, and by 8 weeks lesser than 10 %.

Serum hemagglutinin and hemolysin titers decrease by 3 weeks and after 6 weeks less than 1 : 2 (Fig. 2-C).

TABLE 1 CORRELATION BETWEEN IMMUNOLOGICAL FUNCTION AND CHANGES IN LYMPHATIC ORGANS IN C3Hf/Bi MICE INOCULATED WITH N, N'-DIMETHYLNITROSOUREA

No. of DMNU Inoculation (weeks)		1	2	3	4	5	6	7	8	9	10
Peripheral Blood	White Blood*1 Cell (mm^3)	3150	4560	3900	3600	43002	855	2700	1992	2242	2600
	Per cent of *2 Lymphocyte(%)	67.5	63.5	73	695	9.5	52	61	37.5	63	34
Humoral Response	Serum Hemoylsin Titer	1:128	1:132	1:32	1:32	1:8	<1:2	<1:2	<1:2	<1:2	<1:2
	Serum Hemagglutinin Titer	1:128	1:128	1:64	1:32	1:32	<1:2	<1:2	<1:2	<1:2	<1:2
Cellular Response	Percent PFC in PFC/spleen(%)	121.9	36.5	302	6.6	44.3	17.2	37.5	9.7	11.8	4
	Percent PFC in PFC/ 10^6WBC (%)	123.9	48	49.4	45.1	43.2	29.3	51.2	14.6	17.4	11
Weight of Spleen(gm)*3		.0892	.0829	.1046	.0799	.0921	.0608	.0428	.0512	.0687	.0520
Histological Findings of Thymus	Reduction of Small Lymphocyte	(+)	(+)	(++)	(+++)	(+++)	(+++)	(+++)			
	Appearance of Tumor Cell							(±)	(+)	(+)	(++)

* 1 Average number of WBC in control animals : 5373/mm^3.
* 2 Average per cent of lymphocyte in control animals : 75.5 %.
* 3 Average weight of spleen in control animals : 0.0822 gm.

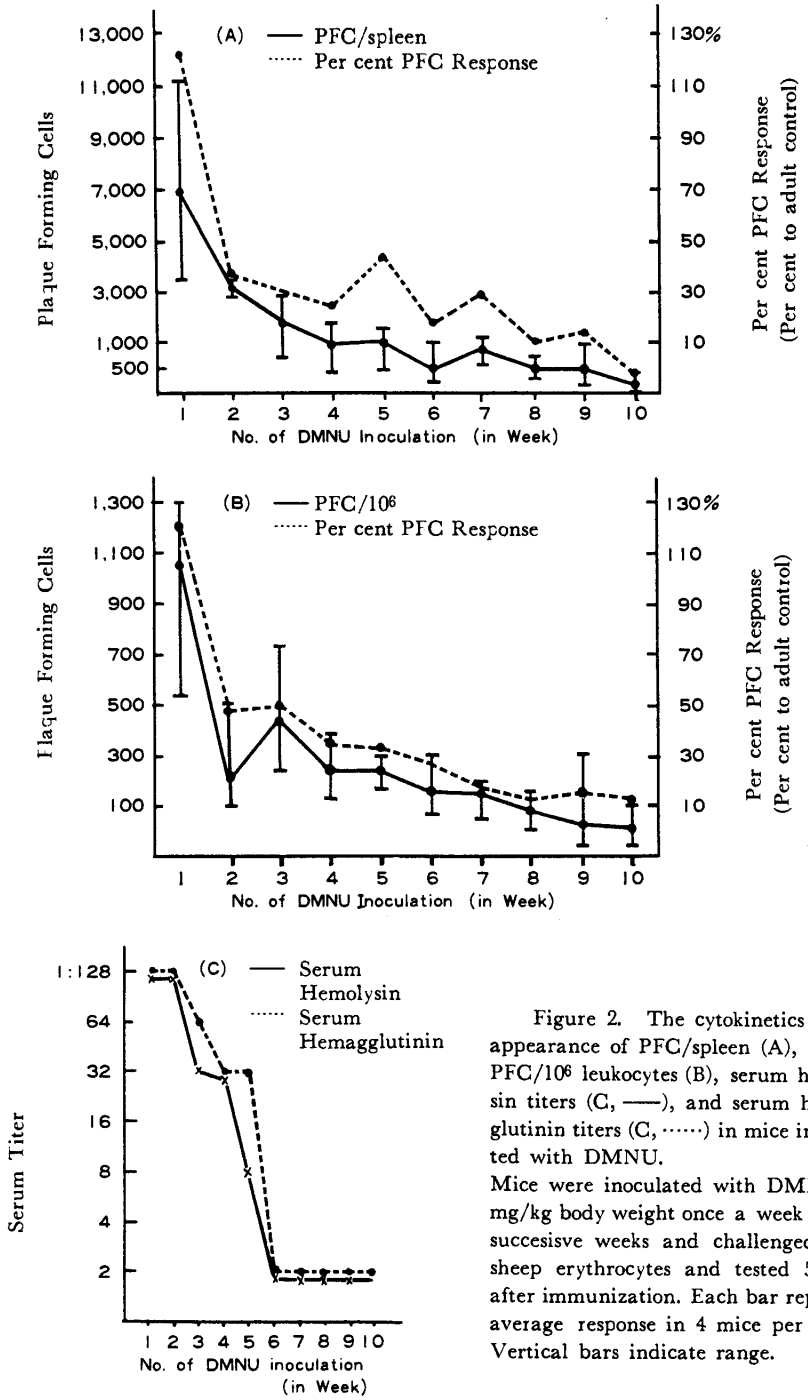

Figure 2. The cytokinetics of the appearance of PFC/spleen (A), PFC/10^6 leukocytes (B), serum hemolysin titers (C, ——), and serum hemagglutinin titers (C, ·····) in mice inoculated with DMNU.
Mice were inoculated with DMNU 80 mg/kg body weight once a week for 10 succesisve weeks and challenged with sheep erythrocytes and tested 5 days after immunization. Each bar repesents average response in 4 mice per group. Vertical bars indicate range.

Effect of N, N'-dimethylnitrosourea on Peripheral Blood

A decreasing tendency in the peripheral leukocyte number was observed as the frequency of N, N'-dimethylnitrosourea injection increased, and after 8 injections it decreased to about half the normal level (Table 1).

Kinetic Changes in the Morphology of the Thymus, Spleen and Lymph Nodes of Mice Inoculated with N, N'-dimethylnitrosourea

Thymus: In continuous observation of the histological changes of the thymus, no qualitative differences could be seen among the mice given the same frequency of injections, but there were quantative differences in the individual cases. The cortical small lymphocytes decreased, and cortico-medullary border became obscure. There could be noticed no marked changes in the medulla (Photos 1, 2, 3, 4, 5 and 6). By 4～5 weeks cortical small lymphocytes decreased more markedly. The medulla showed rather an increase in large lymphocytes as compared with controls, and swelling and proliferation of thymic epithelioid cells was observable (Photo 7). By 7～8 weeks small lymphocytes were hardly recognized in the cortex, and a large number of moderate and large lymphocytes were found. Then, aggregates of lymphoblasts were observed in the cortex and medulla. Later than this stage, cancerous lymphoblasts had gradually prolfierated diffusely both in the cortex and medulla and on the whole giving a monotonous appearance characteristic of malignant lymphoma of the thymus (Photos 8, 9 and 10).

Macroscopically, only one animals showed a tumor formation by 10 weeks. The tumor was of the tip of small finger in size, gray in color and

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Photo 1. Normal thymus ot a control mouse. H-E, × 100.

Photo 2. Cortex of Photo 1; Composing of dense sheet of small lymphocytes. H-E, × 400.

Photo 3. Medulla of Photo 1. H-E, × 400.

Photo 4. Mouse thymus; 2 weeks after DMNU inoculation. Decrease in width of cortex and in concentration of lymphocytes. H-E, × 100.

Photo 5. Cortex of Photo 4; composing of fewer small lymphocytes than Photo 2. H-E, × 400.

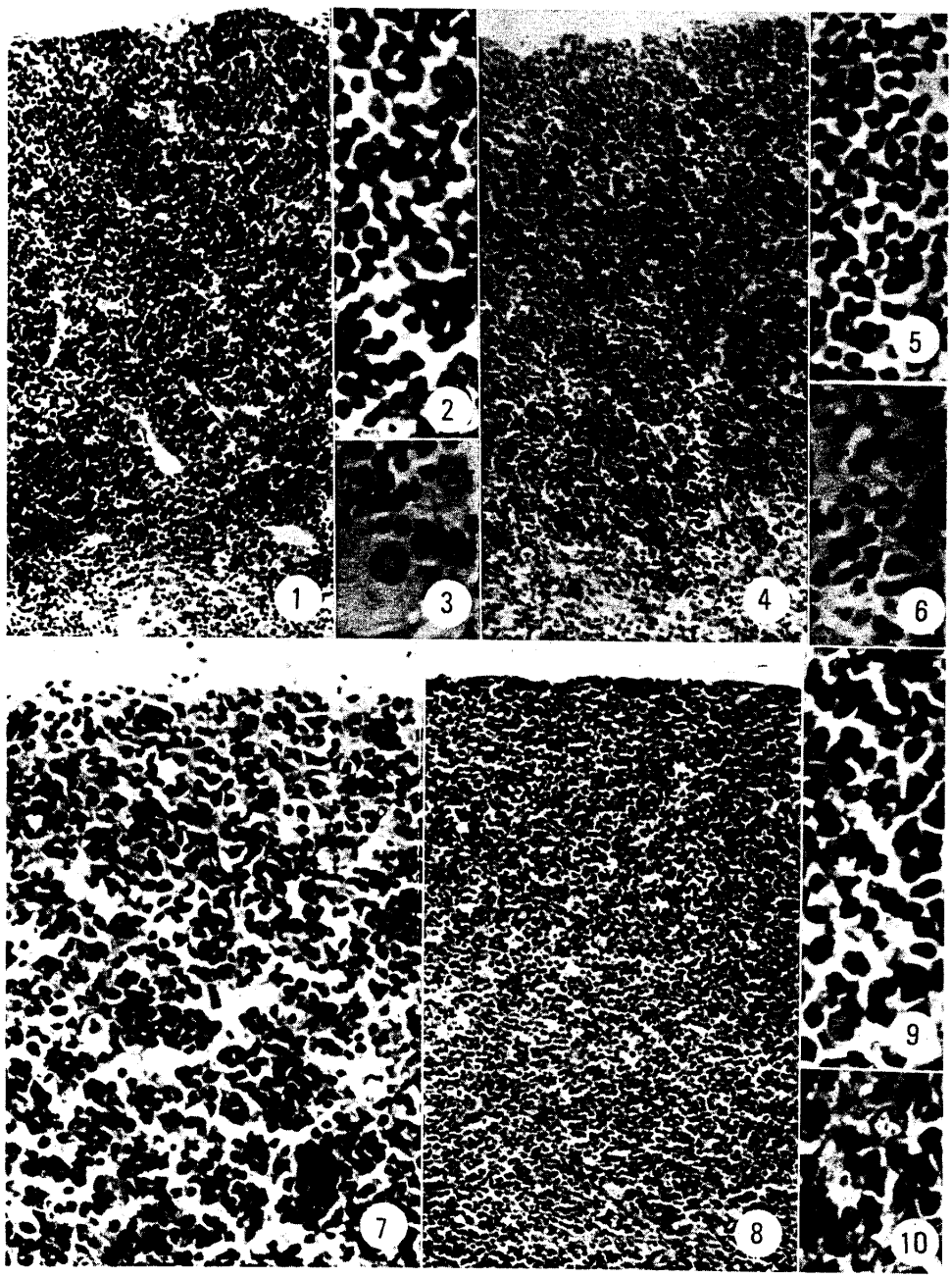
Photo 6. Medulla of Photo 4. H-E, × 400.

Photo 7. Mouse thymus; 5 weeks after DMNU inoculation. Marked reduction in width of cortex, showing obscure border of cortex and medulla with proliferation of large lymphocytes. H-E, × 200.

Photo 8. Mouse thymus; 8 weeks after DMNU inoculation. Moderate proliferaton of lymphoblastic cells with monotonous appearance. H-E, × 100.

Photo 9. Cortex of Photo 8. Proliferation of large tumorous lymphoblasts, H-E, × 400.

Photo 10. Medulla of Photo 9. Lesser proliferation of large tumorous lymphoblasts than in cortex. H-E, ×400.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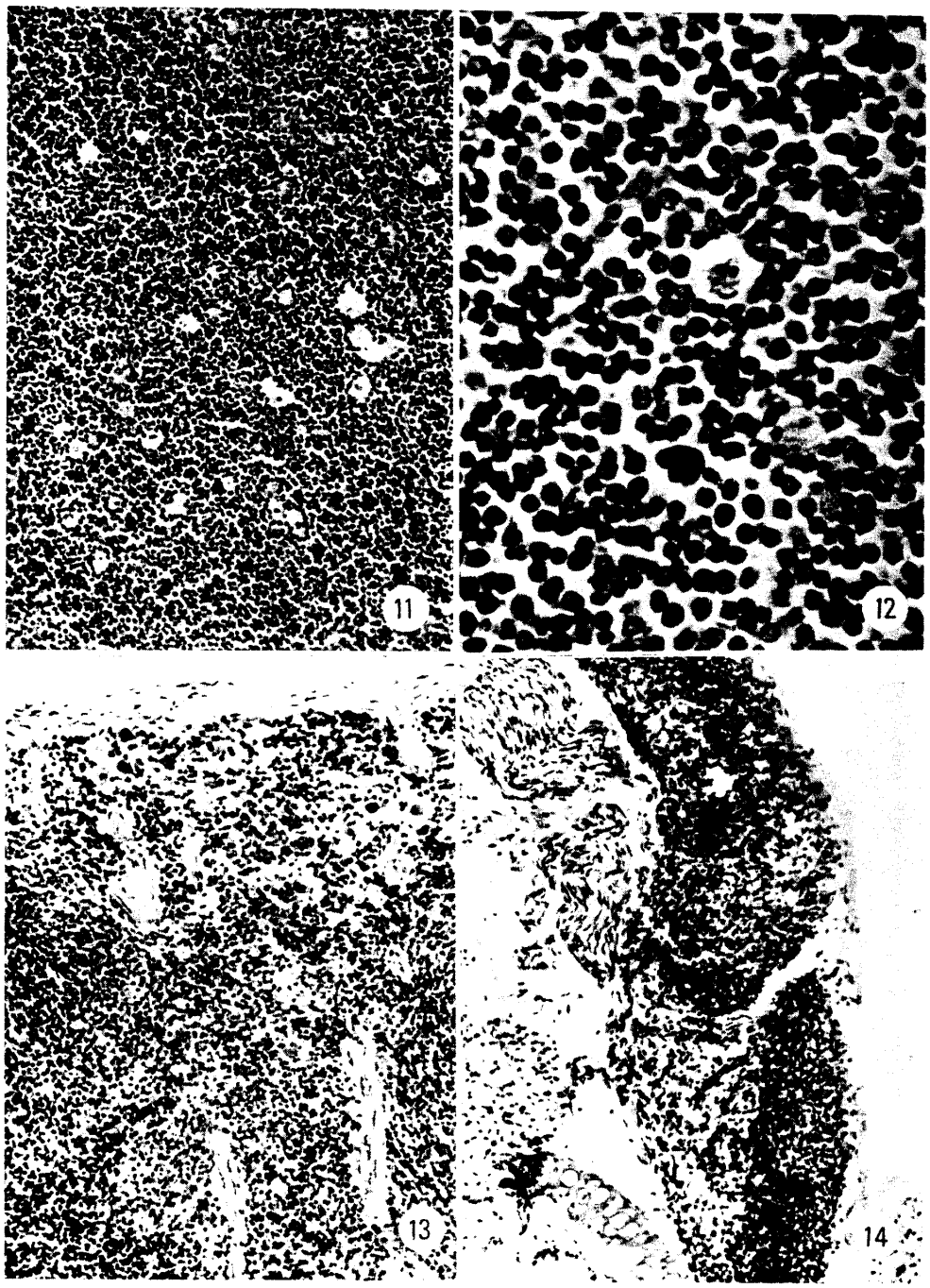
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soft. The tumor was located in the site of thymus and infiltrated into the bronchus and the adjacent structures. Histologically, the tumor was consisted of large cells resembling lymphoblasts accompanied by a considerable number of starry sky cells (Photos 11 and 12).

Spleen: The weight of spleen began to decrease by about 5 weeks, and such a tendency was also observed in total spleen cell count (Table 1). Histologically, the red pulp reduced gradually from the early stage, and then by 7 weeks the lymph follicles were getting smaller corresponding to a decrease of peripheral lymphocyte count without any sign of cell destruction. Such a tendency of lymphocytic reduction developed more markedly and later than 8 weeks fibrosis appeared (Photo 13).

Lymph Node: In comparison to the thymus and spleen, changes in lymph nodes proved to be milder. No remarkable changes could be seen in the structure, but a slight decrease of the lymphocyte was observed by about 8 weeks (Photo 14).

Correlation between Immunological Function and Changes in Lymphatic Organs in C3Hf/Bi Mice Inoculated with N, N-dimethylnitrosourea

Correlation between cellular and humoral responses to sheep erythrocytes on one hand and peripheral leukocyte count, spleen weight and histological changes of the thymus on the other is as shown in Table 1. As the frequency of N, N′-dimethylnitrosourea injections increased, the lymphocytes of the thymus, the leukocyte counts of peripheral blood as well as the weight of the spleen decreased. With the lapse of 8 weeks tumor cells appeared in the thymus. It was found that the antibody production capacity had declined prior to the appearance of tumor cells and further decreased in tumor state.

DISCUSSION

When 80 mg/kg of N, N′-Dimethylnitrosourea is consecutively into C3Hf/Bi mice 5~6 weeks old once every week, there can be observed a formation of malignant lymphoma in the thymus 89 days (13 weeks) in

Photo 11. Tumor of thymus which developed in one animal. Monotonous proliferation of tumor cells accompanied by a considerable number of starry sky cells. H-E, × 100.

Photo 12. Tumor of thymus (the same portion as Photo 11.). Tumor was consisted of large cells reseeming lymphoblast. H-E, × 400.

Photo 13. Mouse spleen; 5 weeks after DMNU inoculation. Slight reduction of lymphocytes. H-E, × 100.

Photo 14. Mouse lymph node; 5 weeks after DMNU inoculation. Slight reduction of lymphocytes. H-E, × 100.

H-E: Hematoxylin and eosin

average (12). We examined histological changes in thymus and lymphatic tissues continuously for the period of 10 weeks after inoculation, which corresponds to the latent period of tumor development, and simultaneously studied the correlation of antibody formation capacity of the host to S-RBC.

As for the histological changes of thymus, a certain decrease in cortical lymphocytes was observed in early stage, and by 8 weeks tumor cells began to make its appearance and then proliferated. These changes were practically similar to these of thymoma induced by x-irradiation (14), viruses (15), or urethan (16), and it seemed to indicate that there occur a destructive change in the thymus and then regenerative proliferation, followed by tumor development. Out of the lymphatic tissue, changes in thymus are most marked, but also in spleen and lymph nodes decrease of lymphocytes corresponding with the course of thymic changes were recognized, and in the terminal stage fibrosis as a repairing process occurred.

Approximately in parallel with histological changes in the lymphatic system, cellular and humonal antibody formation to S-RBC were suppressed. It has been demonstrated that this suppression is to about one third in the early stage of N, N′-dimethylnitrosourea inoculation and progresses to about one tenth at the terminal stage when tumor cells make their appearance. Immunosuppession similar to this system is recognized in the case of thymoma induced by urethan (17), methylcholanthrene (3) or x-irradiation (3). Such an inhibition is known to occur not only in the development of thymoma but also at the onset of leukemia (6, 7, 8) and other tumors (4, 18). It is assumed that in the case treated with chemical carcinogens, viruses, and x-irradiation, both transformation activity and immunosuppressive activity are involved in tumor development.

The mechanism of immunosuppression as observable at the administration of N, N′-dimethylnitrosourea remains yet obscure. However, judging from the fact that N, N′-dimethylnitrosourea has a strong cytotoxicity on thymus, immunosuppression can be thought to occur due to disturbances of thymus function. Even assuming that its mechanism is similar to that of thymectomy, it is said that the effect of thymectomy is relatively mild and temporary in its early stage, and immunosuppression occurs so late as about 9 months after thymectomy (19). As the immunosuppression of N, N′-dimethylnitrosourea to adult mice is stronger and occurs in earlier stage than that of thymectomy, disturbances of the thymus cannot be the sole factor. When total body x-irradiation (20) and administration of cortison (21) or antilymphocyte serum (22) are given

simultaneously with thymectomy, the functions not only of the thymus, but also of the other lymphatic tissues are suppressed, and even in adult animals, immunosuppression is known to occur. The mechanism of immunosuppression by N, N′-dimethylnitrosourea seems to correspond to those of the aforementioned treatments. In other words, as N, N′-dimethylnitrosourea damages not only the thymus but also other lymphatic tissues as well, a strong immunosuppression seems to occur. Histologically also, the decrease of lymphocytes in lymph nodes and spleen seems to be the evidence in support of such an assumption.

Next, in the course of carcinogenesis by N, N′-demethylnitrosourea it remains unclarified what significant role the immunosuppression plays in thymoma development. According to recent studies, tumor cells are said to lose their normal tissues antigens and to acquire the tumor specific antigen(s) (10). In chemical- and virus-induced thymic lymphomas the presence of tumor antigens on the cell surface has been confirmed (23). For cancerization of the cell, it would be necessary for these cells to subdue various resistances of the host, especially important to overcome the immunological resistance. Consequently, the fall in the immunological function of the host would facilitate proliferation of those tumor cells whose antigenicity was changed. However, in the tumor cells induced by N, N′-dimethylnitrosourea it remains unclarified what change of antigenicity has occurred, but it is suggested that in this experimental system immunosuppression plays an impotant role to the tumor development in incidence of 100 per cent. This problems should be further studied.

CONCLUSION

By subcutaneous inoculation of N, N′-dimethylnitrosourea to adult male C3Hf/Bi mice once a week for 10 consecutive weeks the authors studied the correlation between immunological functions and histological changes in lymphatic tissues at the latent period of thymic lymphoma whose development is known to occur in 100 per cent.

As a result, it was found that PFC of the spleen to sheep erythrocytes decreased to about one third the normal level by two weeks, and to one tenth by 8 weeks after initial inoculation of this compound. Hemolysin and hemagglutinin titers of the serum became less than 1 : 2 after 6 weeks and later. As for histological changes in the thymus, disappearance of lymphocytes became marked by 2 weeks, and there appeared tumor cells by 8 weeks. Also the peripheral lymphocytes as well as the total spleen cells decreased in number along with increase of the frequency of inocu-

lation of N, N'-dimethylnitrosourea.

These results seem to suggest that the immunosuppressive effect of carcinogen facilitates the development and proliferation of tumor cells possessing tumor specific antigenicity in the course of N, N'-dimethylnitrosourea-carcinogenesis.

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